kb repeat were L43D (5′-CCG GAA TTC AGA ATT ATA TAT GAG GCT GCG-3′) at nt 66 to 86 with respect to the Hind III site, and L44D (5′-CCG GAA TTC CAA ATA AAG AAA CAT ATT GCG-3′) at nt 2228 to 2248. PCR reactions were performed on total parasite DNA. For detection of the transfection vectors, the PUC forward sequencing primer (5′-TTT TCC CAG TCA CGA CGT-3′) was used in conjunction with either L80R for pMD221, L43D for pMD223, or 530 (5′-GGG GTT CCA TTC AAT ATT GC-3′, which ends at nt 1505 of the DHFR-TS ORF) for pMD200. Oligonucleotide 530 was used in conjunction with the SK primer (5′-TCT AGA ACT AGT GGA TC-3′) designed for pBSKS (Stratagene) for PCR detection of pMD204. The entire DHFR-TS coding region was amplified with the oligonucleotides 644D (5′-GCAGCGGATCC ATG GAA GAC TTA TCT GAA AC-3′, with nt 1 in bold) and 645D (5′-GGGCCGGATCC TTA AGC TGC CAT ATC CAT ATT-3′, which ends at nt 1764). The reaction conditions for all amplifications, except that of the DHFR-TS–pBSKS hybrid (experiment R4), were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. For amplification of DHFR-TS–pBSKS, conditions were identical except that primers were annealed to the template at 45°C. *Taq* DNA polymerase was obtained from Life Technologies. Oligonucleotides L40 (5′-GAA TAC TTT CCC AA**T** TTT TTT TTC-3′, resistant) and L41 (5′-GAA TAC TTT CCC AA**C** TTT TTT TTC-3′, sensitive) were used in hybridization experiments to determine the presence of the resistant and sensitive configurations of the DHFR-TS gene in total parasite DNA. The residues in bold encode the point mutations observed at position 110 in the DHFR-TS protein which determine parasite resistance (L40) or sensitivity (L41) to the drug pyrimethamine. Oligonucleotides were hybridized in 5× standard saline citrate containing 1% SDS at 42°C, and washing was performed at 56°C with 1× standard saline citrate containing 1% SDS for 5 min.

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10. Synchronized erythrocytic merozoites were obtained from mature schizonts, which were isolated from 5 ml of blood obtained by cardiac puncture from a Wistar rat (body mass, 250 g) 5 days after intraperitoneal injection of 10^8 *P. berghei* blood stage parasites (ANKA strain, clone 15cy1, pyrimethamine sensitive). This blood, containing mainly ring- and trophozoite-infected erythrocytes at 3% parasitemia, was incubated under standard culture conditions (RPMI 1640 medium, 20% fetal bovine serum) in a gently shaking Erlenmeyer flask for 20 hours at 37°C. During this time, parasites mature into schizonts that do not rupture and remain viable for prolonged periods. The mature schizonts were separated from uninfected cells by density-gradient centrifugation; the culture was layered on top of a 58% Nycodenz (Nycomed, Oslo, Norway) and phosphate-buffered saline gradient and centrifuged for 30 min at 200*g*. The schizonts accumulate at the interface and were collected and washed once in culture medium. The resulting preparation consisted of 90% mature schizonts and 10% uninfected cells, with a total of 5×10^8 schizonts. These cells were concentrated into 100 μl of RPMI 1640 culture medium and used for electroporation. In experiment R1, 75 μg of plasmid pMD221 and 15 μg of pMD223 (each in the proportion 1:1 of circular to linear forms) were dissolved in 75 μl of TNE buffer (pH 7.2), and the volume was adjusted to 400 μl with phosphate-buffered saline. The DNA and the parasites were mixed, placed in a 0.4-cm electroporation cuvette (Bio-Rad), and subjected to a single pulse (800 V, 25 μF; time constant, 0.8 ms) at room temperature. Earlier experiments showed a 10 to 40% survival rate of merozoites under these conditions. The mixture was then placed on ice for 10 min before intravenous injection into a Wistar rat (250 g). Thirty hours after injection, the rats were treated with a single dose of pyrimethamine (10 mg/kg, intraperitoneally) on each of four consecutive days. In experiment R2, 75 μg of plasmid pMD221 (1:1, supercoiled:linear) was mixed with parasites and the same procedure was followed as for R1. In experiments R3 to R6, 100 μg of pMD200 (R3), 100 μg of pMD204 (R4), 10 μg of pMD223 (R5), or 40 μg of pMD221 (R6) was introduced into merozoites as for R1. Parasites surviving the pyrimethamine treatment were mechanically passaged to naïve mice and rechallenged with pyrimethamine for 3 days as described for rats. Resistant parasites were passaged again to naïve mice for the collection of parasites for nucleic acid isolation and for in vitro drug susceptibility tests. Clones of resistant parasites from experiments R1 and R2 were obtained by limiting dilution and all proved resistant to pyrimethamine when challenged in vivo or in vitro. These clones were grown in naïve mice as before for nucleic acid isolation. Between electroporation of the T1 and T2 parasites and the testing of the resultant drug-resistant T1 clones, a period of 49 days of parasite multiplication had elapsed (including five mechanical passages). The resistance level was established by determination of schizont development in short-term cultures of *P. berghei* (*11*). B. Mons, C. J. Janse, E. G. Boorsma, H. J. van der Kaay, *Parasitology* **91**, 423 (1985); C. J. Janse, B. Mons, J. J. A. B. Croon, H. J. van der Kaay, *Int. J. Parasitol.* **14**, 317 (1984); C. J. Janse, E. G. Boorsma, J. Ramesar, M. J. Grobbee, B. Mons, *ibid.* **19**, 509 (1989).
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18. Supported, in part, by grants from the European Union Science and Technology for Development program (contract TS3*-CT92-0116 to C.J.J.) and the United Nations Development Program–World Bank–World Health Organization Special Programme in Research and Training in Tropical Diseases (contract 930326 to A.P.W.). We thank J. Ramesar for technical assistance; B. Mons and U. Certa for stimulating discussions; M. Ponzi and T. Pace for information on electroporation conditions; and G. van Dam for help with curve "fitting."

13 September 1994; accepted 27 February 1995

Role of the Protein Chaperone YDJ1 in Establishing Hsp90-Mediated Signal Transduction Pathways

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The substrate-specific protein chaperone Hsp90 (heat shock protein 90) from *Saccharomyces cerevisiae* functions in diverse signal transduction pathways. A mutation in *YDJ1*, a member of the DnaJ chaperone family, was recovered in a synthetic-lethal screen with Hsp90 mutants. In an otherwise wild-type background, the *ydj1* mutation exerted strong and specific effects on three Hsp90 substrates, derepressing two (the estrogen and glucocorticoid receptors) and reducing the function of the third (the tyrosine kinase $p60^{v\text{-}src}$). Analysis of one of these substrates, the glucocorticoid receptor, indicated that Ydj1 exerts its effects through physical interaction with Hsp90 substrates.

In vitro, purified Hsp90 can function as a general protein chaperone (*1*). In vivo, however, Hsp90 functions in a large heteroligomeric complex and exhibits a high degree of substrate specificity (*2*). The chaperone activities of Hsp90 participate in a wide range of signal transduction pathways, including pathways controlled by steroid receptors, tyrosine kinases, and serine-threonine kinases (*2*–*7*). These structural and functional properties are highly conserved: Mammalian and yeast Hsp90s are found in similar macromolecular complexes (*2*, *8*), mammalian Hsp90 complements the essential functions of yeast Hsp90 (*3*, *9*), and yeast Hsp90 promotes the activation of steroid-receptor and tyrosine kinase target proteins from vertebrate cells (*3*, *6*, *10*).

To identify proteins functionally related to Hsp90, we searched (Fig. 1) for *Saccharomyces cerevisiae* mutations that would be lethal in combination with Hsp90 mutations that themselves have little effect on growth at 30°C (synthetic-lethal, or *SL*, mutations) (*11*, *12*). To identify the *SL* gene obtained in this search, we screened yeast libraries for plasmids that would complement the *SL* phenotype. All plasmids recovered encoded either Hsp90 or one of two members of the DnaJ chaperone family,

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YDJ1 (isolated as a single-copy plasmid) and *SIS1* (isolated as a multicopy plasmid) (*13*). Because high-copy *SIS1* plasmids suppress *YDJ1* mutations (*13*), it seemed likely that the *SL* mutant was an allele of *YDJ1*. This prediction was confirmed by linkage analysis (*14*). The mutant allele in the *SL* strain was recovered and found to carry a G to A transition in codon 315, changing a highly conserved Gly to Asp in the generally less conserved COOH-terminal region ($ydj1^{G315D}$).

To define further the relation between Hsp90 and Ydj1, we examined the effect of the $ydj1^{G315D}$ (*SL*) mutation on Hsp90 substrates (*3*, *6*, *10*). One of the best characterized of these substrates is the glucocorticoid receptor (GR) (*2*). The chaperone Hsp90 is part of the GR aporeceptor complex and is required to maintain the receptor in a hormone-activatable state (*2*, *3*, *10*, *15*, *16*). When GR is expressed in yeast, Hsp90 mutations strongly reduce the ability of GR to be activated by hormone (*3*, *10*, *16*). We transformed a high-copy GR expression plasmid and a *lacZ* GR reporter plasmid into (i) a wild-type strain, (ii) a strain carrying the $ydj1^{G315D}$ mutation, and (iii) a strain carrying the Hsp90 mutations used in our screen (strain 5CG2 without the *GAL1* promoter substitution) (Fig. 1).

The Hsp90 mutations reduced hormonal induction of β-galactosidase (β-Gal) by about threefold at 30°C (Fig. 2A). The $ydj1^{G315D}$ mutation also affected β-Gal activity, but in a very different way. It increased activity in the absence of hormone by about 200-fold. This increase in reporter-gene expression was specifically due to a change in GR activity. When the reporter plasmid was retained but the GR expression plasmid was lost (by growth on single-selection medium), constitutive β-Gal activity was also lost. With the addition of hormone, β-Gal activity increased in $ydj1^{G315D}$ cells by twofold in 2 hours (Fig. 2A). However, after about 6 hours, when activity had reached a maximum (twice the level observed at 2 hours), $ydj1^{G315D}$ and wild-type cells exhibited equivalent activity (*14*, *17*).

To investigate the reason for increased basal activity in the $ydj1^{G315D}$ mutant, we examined levels of GR protein by immunoblotting. A modest increase in GR accumu-

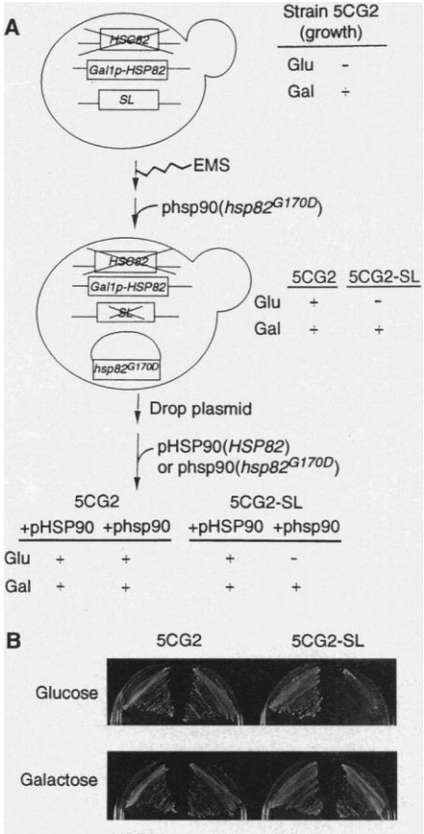

Fig. 1. Synthetic-lethal screen. (**A**) Strategy for obtaining *SL* mutations with a temperature-sensitive Hsp90 mutation (*31*). EMS, ethylmethane sulfonate. (**B**) Growth properties. 5CG2 grows on glucose when transformed with either *HSP82* (HSP90) or $hsp82^{G170D}$ (hsp90 mutant). The *SL* derivative grows on glucose only when transformed with *HSP82*. Cells were streaked on medium without histidine (to maintain the plasmid) and incubated at 30°C for 5 days.

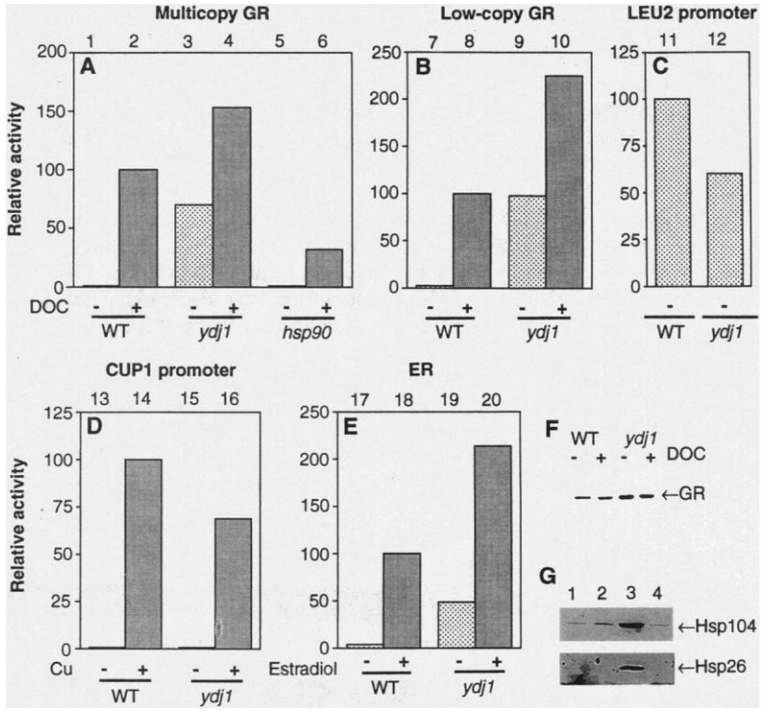

Fig. 2. $ydj1^{G315D}$ selectively increases basal GR and ER activities. Wild-type, *ydj1*, and *hsp90* cells were grown to mid-log phase at 30°C and then treated with or without inducer: (**A** and **B**) 10 μM deoxycorticosterone (DOC, saturating concentration); (**C**) no inducer; (**D**) 50 μM $CuSO_4$; (**E**) 0.1 μM β-estradiol for 2 hours (1 hour for $CuSO_4$). β-galactosidase activity was measured with Galacto-Light (TROPIX) and normalized to total protein concentration as described (*16*). Each point is the average of at least three independent experiments, with two replicates each. Activities are expressed relative to induced activity in wild-type cells. Activities expressed as photon unit per microgram in representative experiments were as follows; 1, 4070; 2, 1434824; 3, 1050795; 4, 2445929; 5, 9170; 6, 434054; 7, 5806; 8, 308314; 9, 330942; 10, 716030; 11, 35581; 12, 20630; 13, 337; 14, 151681; 15, 539; 16, 103897; 17, 5220; 18, 213821; 19, 110935; and 20, 430017. Strains: WT, YPH499 (*32*); *ydj1*, PCy7 (cells harboring the $ydj1^{G315D}$ mutation backcrossed three times to eliminate possible extraneous mutations); and *hsp90*, YOK5ura:HIS (YPH499 cells carrying an *hsc82:HIS3* disruption and a chromosomal replacement of *HSP82* with $hsp82^{G170D}$ (*12*). Plasmids: (A) high-copy GR expression plasmid pGPD-795 (*33*) and *lacZ* reporter pSX26.1 (*33*); (B) single-copy GR expression plasmid pHCA/N795 and high-copy reporter pL2/GZ; (C) *LEU2lacZ* reporter plasmid pYB1 (*34*); (D) *CUP1lacZ* reporter plasmid pCLUC; and (E) high-copy ER expression plasmid p2HGPD/ERcyc (*16*) and *lacZ* reporter pUCΔSS-ERE (*3*). (**F**) Effects of $ydj1^{G315D}$ on GR accumulation. Total proteins were separated on a 10% SDS-polyacrylamide gel, transferred to an Immobilon-P membrane, stained with Coomassie blue to demonstrate equal loading, and reacted with GR-specific antibody, BuGR2 (*16*). (**G**) Effects of $ydj1^{G315D}$ on Hsp104 and Hsp26 accumulation. Total proteins from (lane 1) wild-type (YPH499), (lane 2) *ydj1* (Pcy7), (lane 3) *ssa1ssa2* (A889) (*35*), and (lane 4) wild-type (A812 isogenenic with A889) cells were separated as described in Fig. 2B and reacted with antibody to Hsp104 or Hsp26.

lation was observed in the mutant strain (1.5- to 4-fold in different experiments) [Fig. 2F and (*14*)]. Theoretically, a small increase in GR concentration could produce a large increase in activity, by titrating a limiting repressor. This possibility was eliminated when cells were transformed with a single-copy GR plasmid. The single-copy strains accumulated much less GR protein and exhibited much less GR activity than high-copy strains. Nevertheless, constitutive GR activity was still observed in the absence of hormone in *ydj1*^{G315D} cells (Fig. 2B).

Several experiments demonstrated the specificity of the *ydj1*^{G315D} mutant phenotype for Hsp90 substrates. First, the *ydj1*^{G315D} mutation did not significantly increase the basal activity of *lacZ* plasmids controlled by the *LEU2* promoter or the copper-inducible *CUP1* promoter (Fig. 2, C and D). Second, using antibodies against Hsp104 and Hsp26 (Fig. 2G) and an HSE *lacZ* construct to monitor the stress response (*14*), we detected only a very small increase in hsp abundance as a result of the *ydj1*^{G315D} mutation—far less, for example, than that observed with Hsp70 mutations. Third, analysis of total yeast proteins by means of high-resolution gels revealed that the vast majority of proteins were unaffected by the *ydj1*^{G315D} mutation (*14*). In contrast, mutations in Hsp70 genes (*ssa1ssa2*) changed the pattern of accumulation of several proteins (*14*, *18*). Fourth, when the Hsp90 interaction domain was removed from GR, by deletion of the COOH-terminal region, GR was constitutively active and the *ydj1*^{G315D} mutation had little effect (*19*).

Next, we examined other Hsp90 substrates. Although the estrogen receptor (ER) signaling domain has a primary amino acid sequence that is very different from that of GR, it also binds Hsp90 (*15*, *20*). Moreover, ER activity is reduced by Hsp90 mutations in yeast, albeit not to the same extent as GR activity (*10*). The *ydj1*^{G315D} mutation increased basal ER transcriptional activity by 20-fold (Fig. 2E).

Equally strong effects were observed with a very different Hsp90 substrate, the oncogenic tyrosine kinase p60$^{v\text{-}src}$ (*21*). The maturation of p60$^{v\text{-}src}$ is promoted by Hsp90 (*6*, *16*). Because p60$^{v\text{-}src}$ is toxic in yeast, Hsp90 mutations rescue cells containing a galactose-regulated v-*src* gene from lethality on galactose (*6*). When *pGal10*-v-*src* was transformed into the three test strains, all grew at near wild-type rates on glucose. On galactose, wild-type cells died (Fig. 3A). The *ydj1*^{G315D} mutation rescued growth as effectively as the hsp90 mutations. It also reduced both the accumulation of p60$^{v\text{-}src}$ and its activity as a tyrosine kinase [Fig. 3, C and D, and (*22*)]. Thus, although p60$^{v\text{-}src}$ function was decreased, rather than increased (as observed for GR and ER), it is clear that the function of this Hsp90 target protein was also extremely sensitive to the *ydj1*^{G315D} mutation.

Do the effects of this mutation indicate a role for wild-type Ydj1 in determining the activity of Hsp90 target proteins? A plasmid encoding wild-type Ydj1 restored normal GR regulation in *ydj1*^{G315D} cells (*14*). Moreover, a deletion of *YDJ1* increased basal GR activity to a similar extent as the *ydj1*^{G315D} mutation (*23*), and overexpression of *YDJ1* reduced GR activity, even in the presence of hormone (*14*). Although it is possible that the *G315D* point mutation selectively compromises Hsp90-related functions of Ydj1, the phenotypes of this mutation reflect an important function of wild-type Ydj1.

Ydj1 and Hsp90 are both abundant chaperones (*24*, *25*). To look for a direct association between them, we used a histidine-tagged Hsp90 derivative that allows rapid chromatographic recovery of many Hsp90-associated proteins (*8*). Only a very small fraction of the Ydj1 protein in the cells was recovered, indicating that the two proteins do not form an abundant stable complex (*26*). However, Ydj1 and Hsp90 were both detected in association with an epitope-tagged derivative of GR used to analyze the subfraction of the chaperone proteins that might be associated with substrate (Fig. 4). As expected, Hsp70 (Ssa but not Ssb) was also recovered with GR.

The *ydj1*^{G315D} mutation greatly reduced the association between ydj1 and GR. The capacity of GR to bind to the antibody was also markedly reduced. (Note that in the experiment shown in Fig. 4, the level of GR

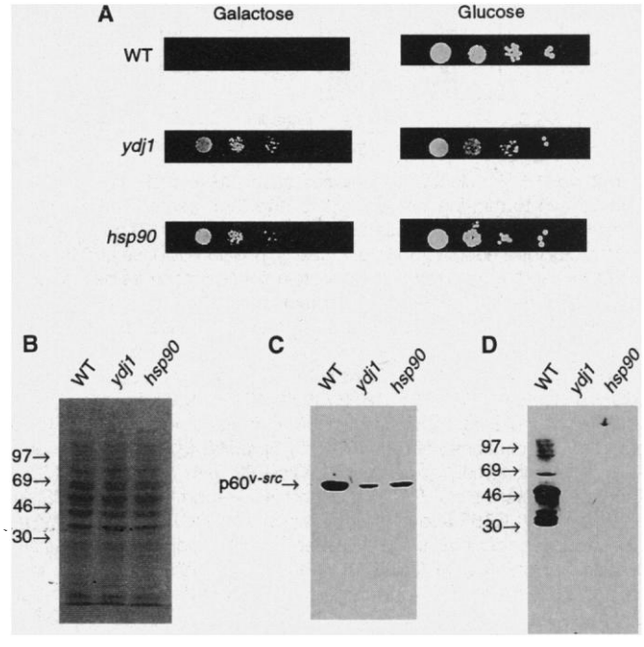

Fig. 3. Effects of *ydj1*^{G315D} and *Hsp90* mutations on p60$^{v\text{-}src}$. (**A**) p60$^{v\text{-}src}$ toxicity. Cells transformed with *pGAL10*-v-*src* were grown to mid-log phase on glucose, adjusted to 10^6 cells per milliliter, serially diluted (10-fold each step), spotted onto synthetic medium, and incubated at 30°C for 4 days (glucose) or 5 days (galactose). *pGAL10*-v-*src* was created by insertion of the *Bam* HI–*Cla* I fragment of *GAL10*-v-*src* (*6*) into pRS313. Strains were as described in Fig. 2, except that YOK5 (*12*) substituted for YOK5ura:HIS. (**B**) General protein accumulation. Total Coomassie blue–stained proteins from mid-log cells induced for 6 hours with galactose and analyzed as in Fig. 2B. (**C**) p60$^{v\text{-}src}$ accumulation. Blot of Fig. 3B, reacted with antibody to v-*src* LA074 (Quality Biotech). (**D**) p60$^{v\text{-}src}$-mediated tyrosine phosphorylation. Blot of (C), stripped, and reacted with antibody to phosphotyrosine (Upstate Biotechnology).

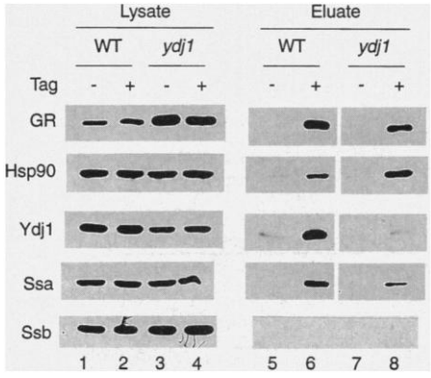

Fig. 4. Specific binding of Hsp90, Ydj1, and Ssa to FLAG-tagged GR. Wild-type and *ydj1*^{G315D} cells were transformed with plasmids encoding an epitope (FLAG)–tagged GR (pCHFLAGGR) (*36*) or wild-type GR (p2HG/N795) together with reporter plasmid pSX.26.1. [In separate experiments examining hormone-inducible β-Gal activity, the epitope-tagged protein was found to be fully functional (*14*)]. Total high-speed supernatants of cell lysates (lanes 1 to 4) and proteins eluted from an anti-FLAG affinity gel (lanes 5 to 8) (*3*, *37*) were separated on SDS-polyacrylamide gels, transferred to Immobilon membranes, and reacted with antibody specific for GR, Hsp82, Ydj1, Ssa, or Ssb. Lanes 1 and 5, wild-type cells, wild-type GR; lanes 2 and 6, wild-type cells, FLAG-tagged GR; lanes 3 and 7, *ydj1*^{G315D} cells, wild-type GR; lanes 4 and 8, *ydj1*^{G315D} cells, FLAG-tagged GR.

was particularly high in the 100,000*g* supernatant of *ydj1*^{G315D} lysates, yet considerably less GR was recovered with the antibody.) Hsp70 recovery also decreased, but only in proportion to the decrease in GR recovery. More surprisingly, Hsp90 recovery increased. Whether this was due to an increase in the strength of the interaction between Hsp90 and GR or to an actual increase in the number of Hsp90 molecules bound to GR is not clear. The simplest explanation of these phenomena is that Ydj1 interacts with GR, and in so doing influences both the conformation of GR and the nature of its association with Hsp90.

The steroid receptor substrates of Hsp90—GR and ER—contain signaling domains with overlapping Hsp90- and hormone-binding regions. These domains serve as autonomous hormone-responsive repressors that can be transferred to other proteins (*15*, *27*). Hsp70 promotes the association of Hsp90 with these domains and Hsp90 helps them to achieve an activation-competent state, such that when hormone binds, the domain adopts a nonrepressing conformation (*2*, *3*, *15*, *28*). The present experiments demonstrate that Ydj1 plays a direct role in helping these domains to achieve their repression-competent state.

Recent work suggests that the complexes formed by receptor and Hsp90 are dynamic, continuously cycling through forms that do or do not contain Hsp70 and other proteins such as p60, p23, and the immunophilins (*2*, *28*–*29*). We suggest that Ydj1 participates in this process, continuously affecting the activity of newly synthesized, previously synthesized, and perhaps even previously activated (recycling) GR (*2*). The activation pathway for p60$^{v\text{-}src}$, in contrast, is direct and does not involve the maintenance of regulated intermediates in the cytosol. Apparently, in affecting interactions between Hsp90 and this substrate, Ydj1 deficiencies simply reduce the level of p60$^{v\text{-}src}$ function in the cell (*30*).

Our *SL* genetic screen and analysis of heterologous Hsp90 substrates have shown a strong relation between Hsp90 and Ydj1 functions. The chaperone Ydj1 participates with Hsp70 and Hsp90 in establishing several signaling pathways, presumably by facilitating critical changes in protein conformation and oligomerization that are required for signal transduction. The participation of the stress-responsive chaperones in these pathways provides additional mechanisms for integrating responses to a wide variety of signals (*5*).

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30. Because p60$^{v\text{-}src}$ is constitutively active in yeast, we cannot determine whether the *ydj1*^{G315D} mutation would increase the basal activity of this class of substrates.
31. In *S. cerevisiae*, Hsp90 is essential for growth and is encoded by two genes, *HSC82* and *HSP82* (*25*). Strain 5CG2, with *HSC82* disrupted and *HSP82* regulated by *GAL1*, can grow on galactose but not on glucose (*12*). Ethylmethane sulfonate–mutagenized cells were transformed with *hsp82*^{G170D}, a temperature-sensitive Hsp90 mutation. The plasmid allowed most transformants to grow rapidly on glucose at 30°C, but those that harbored *SL* mutations could still grow only on galactose. To eliminate extraneous mutations, each *SL* candidate was allowed to lose the plasmid on nonselective galactose medium and was re-transformed with *HSP82* and *hsp82*^{G170D}. Strains that could grow on glucose with *HSP82* but not with *hsp82*^{G170D} were subjected to genetic analysis. One *SL* mutation segregated as a single locus separate from *HSC82* and *HSP82*, caused a temperature-sensitive phenotype in an otherwise wild-type background, and exhibited near wild-type growth at 30°C.
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36. A DNA fragment containing the GPD promoter, an optimal yeast translation-initiation codon, and a FLAG epitope tag coding sequence fused to the NH_2-terminal coding sequence of GR was created by the polymerase chain reaction with oligonucleotides 5′-CCTCGAGGTCGACGGTATCGA-3′, 5′-CTTGTCATCATCGTCCTTGTAGTCCATTTTTCGAAACTAAGTTCTTGGTGT-3′, 5′-ATGGACTACAAGGACGATGATGACAAGGACTCCAAAGAATCCTTAGC-3′, and 5′-GCCCCATGGACAGTGAAAC-3′ [S.N. Ho *et al.*, *Gene* **77**, 51 (1989)]. The fragment was cut with Nco I, Xho I, and Bam HI and ligated to the Nco I–Xho I backbone of pHCA/N795 (CEN plasmid) to create pCHFLAGGR. Because the yeast translation signal promoted high levels of GR expression, a high-copy plasmid encoding wild-type GR was used to control for nonspecific binding.
37. Cells were disrupted with glass beads in buffer A [10 mM MOPS-KCl (pH 7.2), 50 mM KCl, 10% glycerol, 1 mM $MgCl_2$, 2 mM EDTA, 20 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 4-(2-aminoacyl)benzenesulfonyl fluoride (AEBSF), 2 mM benzamidine, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A] plus 2 mM dithiothreitol. After centrifugation for 5 min at 14,000 rpm, fresh PMSF and AEBSF were added, and extracts were cleared twice at 95,000 rpm for 11 min in TL100-2 roto (S. Bohen and K. Yamamoto, unpublished data). Triton X-100 was added a concentration of 0.2%, and equal quantities of total protein were incubated for 1 hour with 100 μl of anti-FLAG M2 affinity gel (5.6 mg/ml) (IBI) equilibrated with buffer A. The affinity gel was collected by centrifugation at 8500 rpm for 1 min and washed (with 5 min of gentle mixing before centrifugation) four times in 1 ml of buffer A containing 0.2% of Triton X-100 and twice with 1 ml of buffer A. Proteins were released with 100 μl of 1× SDS buffer without β-mercaptoethanol and dye (15 min at 4°C).
38. We thank S. Bohen and D. Thiele for providing plasmids; K. Yamamoto, G. Kohlaw, K. Matsumoto, M. Douglas, and E. Craig for materials; and S. Bohen, Y. Miyata, S. Matsumoto, G. Greene, and A. Caplan for helpful discussions. Supported by the Human Frontier Science Program, NIH, and the Ministry of Education, Science and Culture of Japan.

21 November 1994; accepted 21 March 1995